

PLOIDY MANIPULATION IN VACCINIUM SPP.

By

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To overcome crossing barriers and study the genome homology among species of three ploidies in section Cyanococcus of Vaccinium, three approaches were used. The first approach was to take advantage of the relatively high frequency of unreduced gametes in triploid plants, derived from $4x-2x$ interspecific hybridization, to derive hexaploid progeny. A second method was to produce synthetic hexaploid and tetraploid plants via chromosome doubling of triploid and diploid clones. The study of fertility and chromosome behavior in these derived polyploids would then be useful in characterizing species relatedness. A final approach was to screen germinating seeds for twin seedlings in order to derive haploid (polyhaploid) plants, thus allowing for not only the increase, but decrease in ploidy level.

Three triploid blueberry hybrids (V. corymbosum L. ($2n=4x=48$) X V. elliotii Chapm. ($2n=2x=24$)) were crossed reciprocally to hexaploid V. ashei Reade ($2n=6x=72$) and 137 hexaploid F1 plants were derived from pollination of nearly 10,000 flowers. These hexaploid progeny

were presumably the result of $2n$ gamete production. Pollen from the triploids was mostly aborted, and less than 2% were stainable with acetocarmine. However, the triploids did produce from 0.9% to 1.3% unreduced gametes. Meiotic analysis of these triploids revealed trivalents, bivalents, and univalents in all metaphase cells with lagging chromosomes evident at anaphase I. Pollen stainability and pollen germination in the Fl hexaploids were 87.9% and 50.9%, respectively. The number of seedlings produced per flower pollinated with V. ashei pollen in the Fl hexaploids was similar to that obtained from V. ashei X V. ashei crosses. Meiotic analysis of metaphase I and anaphase I and II appeared to be normal.

One synthetic hexaploid (Hex-DT) was produced by in vitro colchicine treatment of triploid FL 81-19. The overall fecundity in Hex-DT was nearly 500 times higher than in the triploid. Pollen stainability and pollen germination in Hex-DT were 42.2% and 13.0%, respectively. Meiotic behavior was highly irregular. In contrast, fertility of an autotetraploid derived from diploid V. elliottii was 50% lower than in the original diploid. The reason for this reduction appeared to be aberrant chromosome behavior during meiosis.

The final experiment consisted of screening seeds germinated in the greenhouse and in vitro to identify sixty-seven pair of twin seedlings. Over 40% of these sets of twins had one weak and one strong member. Chromosome counts allowed identification of three triploids, derived from hexaploid V. ashei, among these twins.

CHAPTER I

INTRODUCTION

The most important problem in employing interspecific hybridization in plant breeding is the low probability of accumulating in one individual the desired combination of genes from two parents. Although obtaining an individual with many desired genes is never an easy task for the breeder, the problem is magnified when wide crosses are used. The process of speciation leads to the development of reproductive isolation barriers that inhibit gene transfer and maintain the integrity of the species.

Interspecific barriers in the broadest sense include all isolation mechanisms between species, such as difference in length of styles and anther filaments; inhibition of pollen germination or pollen tube penetration of the stigma; male sterility or poor flowering of F₁ plants; hybrid breakdown; disturbance of early embryo and endosperm development; and polyploidy. Polyploidy, the multiplication of the chromosome set or genome, is one of the most widespread and distinctive processes affecting the evolution and gene exchange in plant species (Stebbins 1971).

Blueberries (Vaccinium L) are a highly diverse group of many species which exist in almost all parts of the world. Cultivated blueberries are a relatively new crop in the United States, and are little known in many parts of the world. In eastern North America,

many species exist in diverse climates and exhibit ploidy levels ranging from diploid to hexaploid. Several authors (Darrow and Camp 1945; Jelenkovic and Draper 1974; Rousi 1966; Sharpe and Sherman 1971) report no sterility barriers between species with the same ploidy level in the section Cyanococcus of Vaccinium, although Ballington and Galletta (1978) and Vander Kloet (1983) present data to suggest the presence of a weak sterility barrier between diploid species. Heteroploid crosses, on the other hand, can only be achieved with varying degrees of difficulty. Two important reasons for heteroploid crossing are, interspecific gene transfer for plant improvement, and estimation of species relatedness.

Vaccinium ashei Reade (rabbiteye blueberry), a hexaploid species ($2n=6x=72$), is native to the southern United States and has a wide range of adaptability extending from southern Virginia, throughout the coastal plain and piedmont of the Carolinas, and westward to east Texas and Arkansas (Ballington 1981). V. ashei has a number of desirable characteristics for commercial fruit production including resistance to root rot (Phytophthora cinnamomi Rands), cane canker (Botryosphaeria corticis Demaree and Wilcox) (Lyrene and Sherman 1977), high yield, high fruit quality, and a chilling requirement of 400-800 hours of temperatures below 7°C (Galletta 1975). Highbush blueberries (V. corymbosum L) ($2n=4x=48$) are native from central Florida to central Michigan where the lowest temperature expected in an average year ranges from 0°C in Florida to -30°C in Michigan. Cultivars of highbush are widely grown in the South, but several factors restrict their cultivation in Florida. These factors include higher chilling requirement, lack of heat and

drought tolerance, and high susceptibility to certain pathogenic fungi. However, V. corymbosum has two main advantages over V. ashei; early ripening and the larger fruit size. With bloom occurring at approximately the same date, fruits of highbush ripen 20-30 days earlier than fruits of rabbiteye.

In contrast to the hexaploid and tetraploid cultivated species, there are nine diploid species which are not under cultivation. Several of these wild diploid species possess characteristics which could complement the tetraploid and the hexaploid species. A species exhibiting many of the characters needed in highbush and rabbiteye cultivars, including adaptation to less acid soils, early ripening, adaptation to the drier Florida soils, low chilling requirement, high berry flavor, and resistance to certain fungal diseases, is V. elliotii Chapm. (Lyrene 1980).

Attempts have been made to transfer genes between highbush and rabbiteye cultivars. Interspecific hybridization between the two ploidies ordinarily gives rise to pentaploids (Chandler et al. 1985; Jelenkovic and Draper 1973; Vorsa 1987). These are readily produced but, unfortunately, are only partially fertile and progenies from backcrosses of the pentaploids to both rabbiteye and highbush tend to be less vigorous than the pentaploids. Direct crosses between V. ashei and diploid Cyanococcus species are far more difficult to make than V. ashei X V. corymbosum crosses (Darrow et al. 1954; Goldy and Lyrene 1984; Sharpe and Darrow 1959).

Gene transfer between the diploid and the tetraploid species is impeded by the presence of a triploid block. A small number of hybrids have been obtained from this cross, mainly as a result of

unreduced gamete production by the diploid parent (Lyrene and Sherman 1983; Sharpe and Darrow 1959; Sharpe and Sherman 1971). There are several possible methods to bypass the triploid block, including the production of a dihaploid from V. corymbosum, enhancement of unreduced gamete production in the diploid species, and chromosome doubling of the diploid species.

Several strategies have been attempted in order to overcome the ploidy barriers which block or reduce gene movement between diploid, tetraploid, and hexaploid species. Moore et al. (1964) produced a decaploid from a previously produced pentaploid rabbiteye X highbush hybrid. Use of this decaploid has been proposed as a bridge to either 6x or 2x species to produce 8x or 6x breeding lines. These resulting lines, when crossed to 6x or 4x plants would produce 6x clones. Goldy and Lyrene (1984) produced an 8x plant by doubling highbush V. corymbosum, with the intention of backcrossing the 8x plant to the 4x species to produce a hexaploid line that would be based on the highbush genome. To date, these two methods have not been successful for gene transfer. Perry and Lyrene (1984) produced several autotetraploids from the diploid V. ellioti, but the usefulness of these autotetraploids in gene transfer was not evaluated.

The intention of this study was to evaluate specific strategies for overcoming the ploidy barriers that exist between diploid, tetraploid, and hexaploid species of Vaccinium. These strategies include (1) use of the putative triploid clones produced from tetraploid X diploid crosses by Lyrene and Sherman (1983) to transfer traits from the diploid and tetraploid species to the

hexaploid by direct 3x-6x crossing; (2) production of a synthetic hexaploid derived from V. corymbosum X V. elliotii hybrids; (3) evaluation of the usefulness of autotetraploids in gene transfer; and (4) production of haploid and polyhaploid plants from various Vaccinium species.

CHAPTER II

LITERATURE REVIEW

Gene exchange between species in nature is restricted or absent. Without interspecific crossing barriers, species would become submerged in one gene pool. Ecological and geographical isolation of subpopulations of a species may begin the evolution of new species due to genetic drift and natural selection in a different environment. An isolated population may become genetically different from the original population to such an extent that, upon artificial hybridization with the original species, barriers to crossing are evident (Stebbins 1971).

Interspecific hybridization has been used to transfer one or a few genes from one species to another. Other reasons for making interspecific hybridizations would be to determine the relationship of one species to another, to produce new allopolyploid species, to achieve new character expression not found in either parent, to broaden the genetic base, or to provide a bridge between incompatible species (Uhlir 1982; Layne 1983).

Artificial crosses between plant species of the same ploidy level within a genus are often successful, whereas crosses between species of different ploidy levels are difficult or sometimes impossible to perform due to the ploidy barriers to crossing. This type of incompatibility prevents the formation of triploids from

tetraploid X diploid crosses. Marks (1966) first proposed the term "triploid block" when referring to the failure to produce triploids from tetraploid and diploid crosses in potato, where no genic incompatibility is present.

Genic incompatibility is not evident in crosses between diploids and their induced autotetraploids. Woodel and Valentine (1961) presented a series of crosses between diploids and their induced autotetraploids, and observed variation in the expression of the triploid block. It has also been found that interploidy crosses are generally more successful when the species with the higher ploidy level is used as the seed parent (Woodell and Valentine 1961).

In hybrids between allopolyploids and one of the parental diploid species, incompatibility may or may not be genic. Crosses between tetraploid Lamium intermedium and two of its diploid ancestors, L. purpureum and L. amplexicaule, produced no seed regardless of the direction of the cross, whereas the artificially produced autotetraploid of both ancestor diploids produced hybrids rather easily with L. intermedium, indicating that in this situation genic incompatibility is not the problem (Bernstrom 1953). Other examples of this phenomenon include genera such as Gossypium, Rosa, Triticum, and Avena, and have been reviewed by Stebbins (1958).

The interploidy crossing barrier exists in many fruit crop species with varying degrees of strength. In strawberry (Fragaria spp.), diploid species do not cross directly with octoploid species, but cross to hexaploid species via unreduced gametes in the diploid (Evans 1974). In raspberry and blackberry the interploidy barrier

is less evident (Topham 1967). In Citrus species the tetraploid X diploid crosses are easy to make and triploids are viable, but the seed are 3-6 times smaller than the seed produced from tetraploid X tetraploid crosses (Esen and Soost 1973). In apples there are no significant interploidy barriers (Sanford 1983).

Interploidy crosses in Vaccinium represent an example of a very strong triploid block. Despite the fact that one natural triploid clone resulting from a $n + 2n$ fusion has been reported (Ahokas 1971), triploids are extremely difficult to obtain by crossing tetraploid and diploid plants. Crosses between V. corymbosum (4x) and V. darrowi (2x) produce tetraploid hybrids with an average of 52 pollinations being required to obtain one hybrid (Sharpe and Darrow 1959). Childs (1969) also produced tetraploid seedlings when he crossed the hybrids of V. corymbosum X V. australe (4x) with V. pallidum (2x).

Thirty one tetraploid seedlings resulted when 1600 flowers of V. corymbosum were pollinated with pollen from V. darrowi (Sharpe and Sherman 1971). Draper (1977) produced tetraploid hybrids when he crossed V. corymbosum with an interspecific diploid hybrid of V. darrowi X V. atrococcum (2x). It was not until 1983 that triploid seedlings were reported from tetraploid X diploid crosses in Vaccinium (Lyrene and Sherman 1983). The triploids were obtained along with tetraploids, pentaploids, and aneuploids when 7000 flowers of V. corymbosum were pollinated with pollen from V. elliottii (2x).

In addition to the triploid block, other ploidy barriers exist between hexaploids and diploid species. When V. darrowi was crossed

with V. ashei (6x), only five hybrids were obtained from 7500 pollinated flowers (Sharpe and Darrow 1959). These hybrids were vigorous and produced a high percent fruit set when pollinated with tetraploid V. corymbosum. Two of the five hybrids were later examined cytologically and proved to be pentaploid. Previously, Darrow et al. (1954) produced one tetraploid hybrid when V. tenellum (2x) was crossed with hexaploid V. ashei. Draper et al. (1976) crossed V. ashei with V. darrowi and produced a few F₁ hybrids. They suspected the hybrid to be pentaploid due to high sterility. Compared to other interploidy crosses in Vaccinium section Cyanococcus, crosses between hexaploid X tetraploid species or the reciprocal have given the most seedlings. The average number of seedlings per pollinated flower ranges from 1.48 (Chandler et al. 1985) to 2.3 (Lyrene 1988). The seedlings produced from these crosses are partially fertile pentaploids (Brightwell et al. 1955; Brightwell 1966; Darrow 1947; Darrow et al. 1952; Moore et al. 1964; Jelenkovic and Draper 1973; Vorsa 1987).

One factor responsible for the failure of interploidy hybridization is the endosperm impairment which frequently occurs. Therefore, in many cases where homoploid crosses are successful, heteroploid crosses fail because of endosperm abortion. This "ploidy barrier" appears to be involved in failure of crosses between a diploid and its induced autotetraploid, in which no qualitative difference can be suspected, and attempts have been made to explain it in terms of genomic imbalance. Because there are three tissues in close contact within the seed; maternal, endosperm, and embryo, which vary in genetic composition and ploidy,

many researchers have supported the idea that the seed fails to develop when the ploidy of these tissues deviates from normal levels. Muntzing (1933) suggested that a ploidy ratio of 2:3:2 of maternal tissue: endosperm: embryo tissue was required for normal seed development. However, Watkins (1932) found in some cases that seed developed normally with a ratio of 2:6:4, and proposed that only the 3:2 ratio of endosperm:embryo is important in determining viability. Valentine (1954) hypothesized that it was the 2:3 ratio of maternal tissue:endosperm which was important. Stephens (1942) proposed that Gossypium species could be assigned different values or "strength," in order to make their crossing behavior comply to the 3:2 endosperm:embryo ratio.

Some investigators have suggested that it is the endosperm genetic composition that is most essential to interploidy crossing success. Nishiyama and Inomata (1966), working with interploidy crosses in Brassica, hypothesized that the success of endosperm development depends on a 2:1 ratio of the maternal:paternal genomes of the endosperm itself, regardless of the ploidy level of the maternal tissue or the embryo. Lin (1975) supported this idea by his work in maize. He demonstrated that for normal seed development, the endosperm may be of any ploidy level multiple of 3x, provided that the 2:1 maternal:paternal ratio was maintained.

An "endosperm balance number" (EBN) hypothesis has been proposed (Johnston et al. 1980) in order to establish a single unifying concept concerning endosperm function in homo- or hetroloid crosses. Under this hypothesis, the genome of each species is assigned an "effective ploidy" or (EBN) with respect to

endosperm function, by crossing to a species used as a standard. It is the EBN's, not necessarily the ploidies, which must exist in a 2:1 maternal:paternal ratio, for normal development. Excluding any stylar or ovular barriers, two closely related species can be expected to cross if they share the same EBN. Two species with unlike EBN (e.g., EBN=2 and EBN= 4, respectively) can be crossed by first doubling the chromosome number, and consequently the EBN, of the first species. The consistency of this hypothesis has been demonstrated (Arisumi 1982; Johnston and Hanneman 1980; Parrott and Smith 1986).

Recent work on the role of ploidy in endosperm development has been done by Lin (1984) using ig (indeterminate gametophyte), a gene which conditions mitotic abnormalities in the female gametophyte in Zea mays. He was able to produce central cells ranging from 1x to 8x within a 2x female. After crossing igig diploids with females carrying the various ploidy levels obtained, he concluded that neither the ploidy of the maternal parent nor that of the embryo influenced the development of endosperm, but that the genetic constitution of the endosperm itself is important.

The cause of failure in tetraploid X diploid crosses in blueberry species has been explained by the presence of a strong post-fertilization barrier which prevents development of hybrid seed, so that embryo abortion usually occurs before the zygote starts to divide (Munoz and Lyrene 1987).

Unreduced Gametes

A numerically unreduced gamete ($2n$ gamete) is a meiotic product that contains the sporophytic rather than the gametophytic chromosome number. Unreduced gametes may originate from abnormalities during either microsporogenesis or megasporogenesis. They have been demonstrated to function in hybridization of plant species, resulting in the production of higher ploidy level, a process called sexual polyploidization (Mendiburu and Peloquin 1977). Harlan and deWet (1975) reviewed the occurrence of $2n$ gametes throughout the plant kingdom and concluded that sexual polyploidization has been the major route to the formation of naturally occurring polyploids.

Unreduced gametes are probably produced occasionally in most plant species, and most large sexually reproducing diploid plant populations will have occasional sexually derived polyploid individuals (DeWet 1980). In apples, Einst (1945) reported four triploids and three tetraploids among diploid seedlings. In seed from open-pollinated triploids planted with diploid seedlings he also reported ten tetraploids, formed by union of a $2n$ gamete from the triploid plus a $1n$ gamete from the diploid. Crosses in strawberry of $2x \times 8x$ produced $5x$, $6x$, and $9x$ plants depending on which, if either, parent produced $2n$ gametes (Bringhurst and Senanayake 1966). Olden (1965) reported $4x$, $5x$, $6x$, $7x$, and $8x$ progeny resulting from $2x-6x$ interspecific crosses in Prunus. Unreduced gametes have also been reported in pear (Dowrick 1958), citrus (Esen and Soost 1972), raspberry (Pratt et al. 1958), blackberry (Aalders and Hall 1966) and in many agronomic crops such

as potato (Hanneman and Peloquin 1967) and alfalfa (Bingham and McCoy 1979).

Although $2n$ gametes can arise in fertile euploid species, certain unusual situations can select for them almost exclusively. The irregular chromosome association during the meiotic division of odd-ploid plants generally results in nearly all aneuploid gametes. Because of the aneuploid gametes are nonfunctional, the euploid unreduced gametes may represent a relatively high fraction of the functional gametes. Endosperm imbalance may eliminate triploid zygotes formed after $4x.2x$ crosses, whereas tetraploid zygotes formed with $2n$ gametes from the diploid parent survive (Johnston et al. 1980).

Members of nearly all blueberry species produce unreduced gametes at low but significant frequencies (Cockerham and Galletta 1976; Megalos and Ballington 1987). The frequency of $2n$ gametes also varies from species to species and from clone to clone (Cockerham and Galletta 1976). Production of $2n$ gametes has been observed in crosses between tetraploid and diploid species, giving rise to tetraploid progenies as a function of unreduced gametes by the diploid species (Childs 1966; Draper 1977; Lyrene and Sherman 1983; Sharpe and Darrow 1959; Sharpe and Sherman 1971).

A quick method of estimating the frequency of male unreduced gametes is based on visual discrimination of stained $2n$ and $1n$ pollen. In potato, $2n$ and $1n$ pollen have a diameter range of 18-23 μ m and 26-33 μ m, respectively. A positive correlation between frequency of large pollen and seed set from $4x.2x$ crosses has been reported by Jacobsen (1980). Blueberry pollen is normally shed in

tetrads. Thus, $2n$ gametes from abnormal meiosis occur as monads, diads, and triads. The increase in pollen size has also been an effective indicator for the presence of $2n$ gametes in blueberry (Cockerham and Galletta 1976).

The frequency of $2n$ gametes is subject to a large variability caused by the micro-environmental (within the anthers) and macro-environmental variation. Ramanna (1979) found large intra-clonal variation for percentage of $2n$ gametes, ranging from 12.4 to 75.1%, when he sampled several clones of Solanum phureja on different dates in 4-6 successive years. Mok and Peloquin (1975) hypothesized that $2n$ gamete production is controlled by one recessive gene, ps, while Veilleux and Lauer (1981) hypothesized an incompletely penetrating gene with variable expressivity. The substantial influence of environmental conditions on the occurrence and frequency of $2n$ gametes in most genotypes complicates genetic analysis. On the other hand, it offers a possibility of exploiting environmental variation to enhance $2n$ gamete frequencies or even to induce their formation.

Several attempts have been made to induce $2n$ gamete formation with the application of experimental treatments to plants during meiosis. Lewis (1943) applied heat treatment (40° - 46°C) to branches of fruit trees during bud formation and obtained triploid pears following self-pollination of a self-incompatible diploid. Low temperatures or chloroform treatments enhanced the production of $2n$ gametes in Raphanobrassica. The frequency of $2n$ gametes was also enhanced in Brassica oleracea by high temperature, delayed pollination, or application of gibberellic acid to the bud (Eenink

1974). Application of colchicine to immature flower buds has resulted in giant pollen grains with polyploid chromosome numbers in different Prunus species (Olden 1954), Allium (Levan 1939), Beta (Rasmusson and Levan 1939), wheat (Dover and Riley 1973), and rye (Bowman and Rajhathy 1977; Puertas et al. 1984).

Induced Chromosome Doubling

Chromosome doubling can be induced by natural or artificial conditions which disrupt cell division. Natural chromosome doubling has been observed in many crops such as apple (Einset and Imhofe 1951), grape (Ourecky et al. 1967), and citrus (Barrett and Hutchinson 1978). Artificial chromosome doubling can be achieved by using a number of chemical or physical agents such as bromo-acenaphtene (Shmuck and Kostoff 1939), nitrous oxide (Kasha 1974), colchicine (Ackerman and Dermen 1972; Blakeslee and Avery 1937; Chen and Goeden-Kallemeyn 1979; Dermen 1937, 1940, 1945, 1947, 1954, 1967; Draper et al. 1971; Goldy and Lyrene 1985; Lyrene and Perry 1982; Moore et al. 1964; Perry and Lyrene 1984), temperature shock (Dermen 1938), and severe pruning to encourage adventitious bud break (Janick and Moore 1975). The greatest success however, has been with colchicine.

The effect of colchicine is to paralyze the formation of spindle fibers in mitosis. Without a spindle, the cell plate fails to form, so that chromosomes and their duplicates remain in the same cell. Thus, the dividing cell begins diploid and ends tetraploid. Colchicine affects the dividing cells for as long as it remains in contact with the cell. When the colchicine is removed, the cell resumes a normal pattern of division.

Colchicine is usually applied to germinating seeds, seedlings, or rapidly growing shoots and buds. Colchicine can be applied by various methods. It has been dissolved in water, or incorporated into glycerine or a lanolin paste at concentrations ranging from 0.01 to 1%. The duration of treatment has ranged from just wetting the plant tissues to soaking for over 24 hours (Dermen 1940). Only a portion of the cells in a plant undergo chromosome doubling since not all cells are actively dividing at one time; this can result in different ploidy levels or chimeras (Dermen 1967).

Chromosome doubling increases cell volume. This increase extends to nearly all determinate parts of the plant, such as leaf size and thickness, flower and fruit size (Sanford 1983). In general, a newly doubled plant has a more rugged appearance, looks sturdier and has certain giant-like features. Usually growth is slower and the plant is shorter than the original undoubled plant (Sanford 1983).

Two common disadvantages of chromosome doubling are reduced pollen production and reduced female fertility. It has been suggested, for example, that the sterility and lowered seed set may be due to the inviable, unbalanced gametes resulting from the misdisjunction of multivalents formed at meiosis in newly induced polyploids (Darlington and Mather 1949). Other meiotic abnormalities like lagging chromosomes at anaphase I and II or spindle abnormalities have been suggested as probable causes for lower seed fertility (Jackson and Casey 1980). Kostoff (1940) cited examples of species with smaller chromosomes and less quadrivalent formation exhibiting a lower degree of sterility after chromosome

doubling than species with larger chromosomes.

Colchicine induction of chromosome doubling has been successful with many herbaceous species (Dewey 1980). Woody perennial species, on the other hand, have yielded limited success through conventional methods of colchicine treatment (Darrow 1949). Grape (Fry 1963), pear (Janick and Moore 1975), azalea (Pryor and Frazier 1968), cranberry (Dermen 1945), chestnut (Dermen and Diller 1962), camellia (Ackerman and Dermen 1972), and blueberry (Aalders and Hall 1963, Moore et al. 1964) have been doubled with limited success.

Doubling the chromosome number of plants has been done for many reasons. The most valuable has been to restore fertility in wide hybrids (Ackerman and Dermen 1972; Arisumi 1973, 1975; Jones 1970; Semnuik 1978) due to restoration of homologous pairing. Induced polyploids have been used as a bridge to transfer genes between two plants with unequal chromosome numbers (Dewey 1980), and to enhance the crossability of species (deWet 1980).

Haploidization

Haploids of higher plants are individuals or tissues that have somatic cells with a gametic chromosome number. They have been reported in many plant species, and it is likely that they may occur spontaneously in all species at variable frequencies. Haploids from diploid species are referred to as monoloids and have the $1n$ chromosome number, whereas those derived from polyploids are referred to as polyhaploids.

During the last two decades breeders have become interested in the production of haploids for many reasons. The production of haploid plants followed by chromosome doubling results in immediate

homozygosity (Brown and Wernsman 1982). Haploids are suitable as basic material for building monosomic series, useful in cytogenetics and plant breeding (Sears 1954). Because there is no allelic dominance in monoploids, they can be utilized in mutation breeding since the genotypes of such plants are reflected by their phenotypes (Abel 1955). Haploids can be used to transfer genes from tetraploid to diploid species (Peloquin et al. 1966). Additionally they have proven useful in evolutionary studies. An example deals with the evolution of the tetraploid cultivated potato Solanum tuberosum. By obtaining the dihaploid and contrasting it with the tetraploid, Howard (1973) was able to determine that Solanum tuberosum is not a full autotetraploid.

Haploids can originate spontaneously or by induction. Haploid sporophytes are often found sporadically in a population with variable frequency (Kasha 1974). Spontaneous haploids have been reported consistently in flax (Kappert 1933), maize (Chase 1949), potato (Hougas et al. 1964) and cotton (Turcotte and Feaster 1963). Spontaneous haploids can originate by three processes: (1) from the unfertilized egg cell (parthenogenesis), (2) from the male gamete or sperm nucleus (androgenesis), and (3) from any haploid cell of the embryo sac other than the egg cell, specifically, the synergid or antipodal cell (apogamety) (Kasha 1974). The production of haploids from egg cells or synergids is much more frequent than from sperm nuclei or antipodal cells (Lacadena 1974).

Polyembryonic seeds are a source of spontaneous haploids. Frequently one or more embryos are not fertilized but their development is stimulated by the presence of the pollen (Lacadena

1974). Twin seedlings occur with a low frequency in many plant species. Twin seedlings have been considered a source of haploids in more than forty two species which represent about 18% of the total haploids observed to date (Kimber and Riley 1963). Different rates of haploidy ($n-n$ or $n-2n$) are found in the polyembryonic seeds of different species. For instance, Morgan and Rappleye (1950) found 30% $n-2n$ twins among pepper polyembryonic seeds, while Wilson and Ross (1961) found only 5% $n-2n$ in common wheat. In Triticum durum, Sendino and Lacadena (1974) found a frequency of polyembryony of 0.037% and 0.047% among 78,922 and 77,182 seeds of the cultivars Senatore Capelli and Bidi 17, respectively. The proportions of $n-2n$ twins were 10.3% and 5.6% respectively.

Haploidy can be induced by several methods. The most commonly used physical agents for haploid induction are irradiation with x-rays (Swaminathan and Singh 1958), gamma rays, radioisotopes, and various forms of stress, including injury and temperature shock (Lacadena 1974). Various chemicals have been used in the development of haploid plants. The most promising of these chemicals are those that inactivate the sperm nucleus without preventing the growth of the pollen tube, resulting in stimulation of division of the egg cell without fertilization. Toluidine blue is an agent which prevents the division of the generative nucleus in the developing pollen tube (Al-Yasiri and Rogers 1971). Spermatoc nuclei have also been induced to divide and develop into haploid plants by treatment with nitrous oxide (N_2O) (Montezuma de Carvalho 1967).

Interspecific hybridization has been used to produce haploids

in a few species, including barley, potato, and alfalfa (Rowe 1974). Kasha and Kao (1970) crossed cultivated barley, Hordeum vulgare ($2x=14$), with a wild relative, H. bulbosum ($2x=14$), and were able to produce a large number of barley haploids. The haploids result from fertilization and subsequent elimination of the H. bulbosum chromosomes in the developing embryo. It has been suggested that chromosome elimination results from asynchronicity in the mitotic cell cycle in the parental species (Kasha 1974). Ho and Kasha (1975) have shown, through the use of trisomics, that chromosome elimination in hybrids of H. vulgare X H. bulbosum is controlled by genes on chromosome 2 and 3 of H. vulgare.

Other crosses between barley species have also resulted in a high percentage of haploids. Rajhathy and Symko (1974) crossed H. lechleri ($6x$) and H. vulgare ($2x$) and obtained about 50% haploid plants. Crosses between H. jubatum ($4x$) and H. bulbosum ($2x$) produced only haploids, all of maternal origin.

Intergeneric crosses between Triticum ventricosum and H. bulbosum have resulted in 8% haploid plants of the Triticum genotype. Other Triticum haploids have also been produced through hybridization with H. bulbosum, including T. aestivum and T. crassum (Fedak 1982).

A high incidence of haploidy has been reported in potatoes following crosses between Solanum tuberosum ($2n=4x=48$) and S. phureja ($2n=2x=24$). The production of haploids in potato has been popular since they permit breeding at the diploid level (Peloquin et al. 1966)

The discovery of a method for producing haploid plants from

anther culture by Guha and Maheshwari (1964) increased the potential for using haploidy in breeding. The technique is relatively simple. Anthers are cultured on a medium in which conditions are adjusted so that only pollen is induced to divide. The pollen cells can either develop directly into embryoids or develop into disorganized callus from which plantlets are derived. The response of anthers placed in culture is largely dependent on the plant genotype. Anthers containing pollen cells at an optimum stage of development must be used for successful production of haploid plants. Pollen cells at or just following the first pollen mitosis produce most success in culture in many species (Collins 1977). Haploid plants have been developed from anther or pollen cultures in at least 124 species representing 27 families (Bajaj 1983).

CHAPTER III

PRODUCTION AND VIABILITY OF UNREDUCED GAMETES IN TRIPLOID INTERSPECIFIC HYBRIDS

Introduction

The cultivated blueberries of North America are of three major types: lowbush, highbush, and rabbiteye. These correspond loosely to three species in Vaccinium section Cyanococcus: V. angustifolium Ait., V. corymbosum L., and V. ashei Reade respectively. Interspecific hybridization has been used in cultivar breeding, especially among highbush blueberries. Section Cyanococcus also contains many uncultivated species (Camp 1945).

Vaccinium section Cyanococcus appears to be evolving rapidly. Some of the species that differ markedly in habitat preference and in morphology can readily be hybridized in the greenhouse, and form vigorous, fertile hybrids (Darrow et al. 1952, Ballington and Galletta 1978, Vander Kloet 1983). Two factors that reduce natural interspecific hybridization among sympatric species are differences in habitat preference and differences in chromosome number (Camp 1945, Darrow and Camp 1945, Galletta 1975).

Success rates from heteroploid crosses range from moderate to very low depending upon the species and ploidy levels involved. Possibly the most successful heteroploid cross attempted to date is V. corymbosum (4x) x V. ashei (6x), or the reciprocal cross, which yields partially fertile pentaploids (Moore et al. 1964, Jelenkovic and Draper 1973, Vorsa et al. 1987). Crosses between tetraploid and diploid species yield mostly tetraploid hybrids (Sharpe and Darrow

1959), and the ease with which the cross can be made varies directly with the frequency of $2n$ gametes produced by the diploid parent. Frequency of $2n$ gamete formation varies widely among Vaccinium species and among clones within species (Cockerham and Galletta 1976, Megalos and Ballington 1987).

The triploid block, which prevents recovery of triploid hybrids in tetraploid x diploid crosses (Woodell and Valentine 1961), is strong in Vaccinium. Until recently, the only triploid reported in the genus was a naturally-occurring clone of V. vitis-idaea L. found in Finland (Ahokas 1971). Attempts to enhance production of hybrid seedlings from tetraploid V. corymbosum x diploid V. elliotii crosses by various in vitro techniques were not successful (Munoz 1985).

As a result of numerous attempts to cross tetraploid highbush V. corymbosum cultivars with the native diploid species V. elliotii, we obtained three vigorous triploid hybrids (Lyrene and Sherman 1983). The purpose of this study was to examine the fertility of these hybrids, particularly in crosses with hexaploid V. ashei.

Materials and Methods

The three triploids examined in this study were obtained from a population of 300 seedlings produced by pollinating 7000 flowers of tetraploid breeding lines from the University of Florida blueberry breeding program with pollen from the diploid wild species V. elliotii. About fifteen different tetraploid clones were used as seed parents. The three triploid hybrids were derived from three different tetraploid parents: Fla. 78-15, Fla. 65-12, and Fla. 64-76. The three triploid clones were identified by counting

chromosomes from somatic cells of thirty five plants that appeared to have hybrid characteristics.

Meiosis was studied in the three triploids. In order to estimate the frequency of unreduced gametes in the three triploid clones, pollen diameter and stainability were determined by microscopic examination after staining one hour with acetocarmine. Considering only the well-strained pollen, the frequency of unreduced gametes was estimated using the equation

$$A + 2B + C$$

$$T$$

where A is the number of monads, B is the number of diads, C is the number of triads, and T is the total number of pollen grains examined. Fertility of the three triploid clones was estimated by crossing them with hexaploid V. ashei cultivars and by intercrossing and self-pollinating the triploids. F₁ seeds were extracted from mature berries, dried and refrigerated until late October, and then germinated on the surface of peat in the greenhouse. Seedlings were transferred to the field the following May. Of the 165 seedlings obtained, 111 were selected as hybrids based on vegetative, flower and fruit characteristics. Flower buds from the hybrid plants were collected for chromosome counts.

Results

Chromosome associations at metaphase I were similar for the three triploid clones and included univalents, bivalents, trivalents and quadrivalents (Figs. 3-1 a and 3-1 b). Table 3-1 shows the various associations observed. Anaphase I frequently showed one to six

Fig. 3-1. Meiosis in triploid FL 82-208 ($2n=3x=36$). a. metaphase I with 5 I, 4 II, 5 III, and 2 IV. b. metaphase I with 8 I, 4 II, and 4 III (arrow). c. anaphase I with 2 lagging chromosomes. d. late anaphase II with no lagging chromosomes.

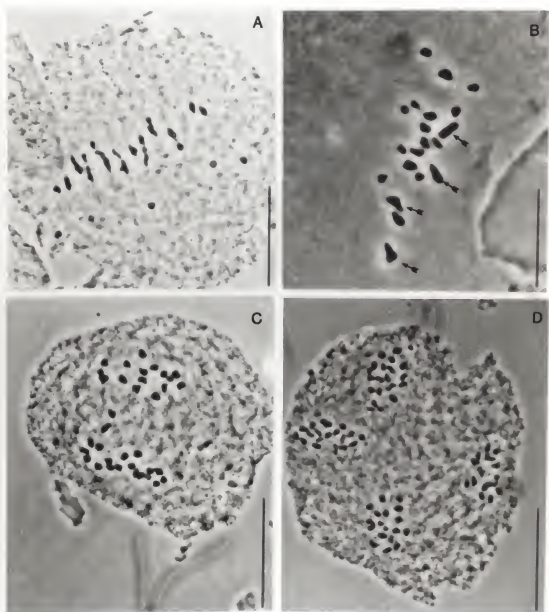


Table 3-1. Range and mean of chromosome associations in PMCs of triploid blueberry at Metaphase I.

clone	No. of cells	Chromosome associations at Metaphase I			
		Univalents	Bivalents	Trivalents	Quadrivalents
80-1	15	4-9 (7.52)	5-10 (8.00)	2-5 (3.09)	0-2 (0.80)
81-19	11	5-9 (7.59)	4-10 (6.90)	3-6 (3.02)	0-2 (1.39)
82-208	25	3-8 (6.00)	5-9 (6.60)	2-6 (3.64)	0-2 (1.47)

lagging chromosomes which were maintained through telophase I (Fig. 3-1 c). Very few lagging chromosomes were evident in anaphase II (Fig. 3-1 d). Less than 1.5% of the pollen in each of the three triploid clones was stainable using acetocarmine. Most of the pollen was small, irregularly shaped, and apparently abortive. Each of the three triploid clones produced 0.9% to 1.3% large, well-stained microspores, occurring as monads and dyads (Table 3-2). These were assumed to contain $2n$ or $4n$ gametes.

Fertility of these triploids, measured as percent fruit set, number of large seed per fruit, and percent seed germination, was very low in all crosses attempted (Table 3-3). Hexaploid V. ashei cultivars pollinated with pollen from the three triploids produced 0.6% to 5.0% fruit set and averaged fewer than two full-size seeds per fruit (Table 3-3). Compared to the average fruit set percentage (46) and the average number of seed per berry (9) in hexaploid x hexaploid crosses (El-Agamy et al. 1981), fertility of hexaploid x triploid crosses was approximately 1.2% as high. Only one triploid clone, Fla. 82-208, set fruit when pollinated by hexaploid V. ashei, whereas the other two clones showed complete female sterility regardless of pollen source. Intercrosses among the three triploid clones and self-pollinations also failed to set seed except with Fla. 82-208. All seedlings from V. ashei x triploids were hexaploid, as determined by chromosome counts, whereas triploid x hexaploid, triploid x triploid, and self-pollination of triploids produced progenies with chromosome numbers ranging from 60 to 72 (Table 3-4).

Table 3-2. Frequency of sporad types and estimated unreduced gamete frequency for 3 triploid clones.

Triploid clone	Total examined	Number of sporads ^a consisting of				Estimated unreduced gamete frequency
		One large stained spore	Two large stained spores	One large stained spore plus 2 small spores	Estimated	
Fla. 80-1	7024	6721	32	61	210	1.3%
Fla. 81-19	2170	2070	4	15	61	1.1%
Fla. 82-208	3854	3741	12	25	76	0.9%

^a A sporad comprises the post-meiotic products of one pollen mother cell which in Vaccinium are bound together as a unit.

Table 3-3. Fertility of blueberry triploids and viability of resulting seeds and progeny.

Seed parent	Pollen parent	Flowers pollinated	% Fruit set	Mean plump seed/fruit	No. of seedin	No. of hybrids ^z
<u>V. ashei</u> cultivars (6x)	Fla. 82-208 (3x)	5206	0.6	1.8	23	16
<u>V. ashei</u> cultivars (6x)	Fla. 81-19 (3x)	2311	3.0	1.9	42	22
<u>V. ashei</u> cultivars (6x)	Fla. 80-1 (3x)	3336	5.0	1.6	74	53
Fla. 82-208 (3x)	<u>V. ashei</u> (6x)	809	10.5	1.7	61	61
Fla. 81-19 (3x)	<u>V. ashei</u> (6x)	2160	0.0	0.0	0	0
Fla. 80-1 (3x)	<u>V. ashei</u> (6x)	3174	0.0	0.0	0	0
Fla. 82-208 (3x)	Fla. 81-19 (3x)	717	0.0	0.0	0	0
Fla. 81-19 (3x)	Fla. 82-208 (3x)	390	3.3	1.0	2	2
Fla. 81-19 (3x)	Fla. 80-1 (3x)	1711	0.0	0.0	0	0
Fla. 80-1 (3x)	Fla. 82-208 (3x)	1270	0.0	0.0	0	0
Fla. 80-1 (3x)	Fla. 81-19 (3x)	997	0.0	0.0	0	0
Fla. 82-208 (3x)	Self-pollinated	810	0.6	1.0	3	0
Fla. 80-1 (3x)	Self-pollinated	800	0.0	0.0	0	0
Fla. 81-19 (3x)	Self-pollinated	919	0.0	0.0	0	0

^z Showed elements of parent species morphology.

Table 3-4. Distribution of chromosome number in progeny from crosses using blueberry triploids as male and female parent.

Cross		No. of progeny	Chromosome no.					
Female parent	Male parent		72	71	70	69	68	60
<u>V. ashei</u> (6x)	Fla. 80-1 (3x)	53	51		2			
<u>V. ashei</u> (6x)	Fla. 81-19 (3x)	22	22					
<u>V. ashei</u> (6x)	Fla. 82-208 (3x)	16	16					
Fla. 82-208 (3x)	<u>V. ashei</u> (6x)	61	35	9	9	3	2	3
Fla. 82-208 (3x)	Fla. 80-1 (3x)	2	1	1				
Fla. 82-208 (3x) ^z	Fla. 82-208 (3x)	3	2		1			

^z Manual self pollination.

Discussion

The fact that few triploids have been reported from large-scale 4x-2x crossing efforts in Vaccinium, despite the recovery of the fairly large number of 4x hybrids (Sharpe and Darrow 1959, Sharpe and Sherman 1971), indicates that the triploid block is well developed in Vaccinium. Selection pressure favoring the evolution of such a block would probably be high in nature due to the frequent sympatric occurrence of diploid and tetraploid Vaccinium species (Camp 1945, Vander Kloet 1977, Lyrene and Sherman 1980), coupled with the high degree of sterility observed in triploids. The recovery of triploid hybrids from tetraploid V. corymbosum x diploid V. elliotii crosses probably does not reflect a weakening of the triploid block with this species combination, but results instead from the very large number of flowers that were pollinated, along with the relatively low frequency of tetraploid hybrids produced.

The three triploids studied were similar in meiotic behavior although there was considerable variation in seed set. The high frequency of trivalents (2-6 per meiocyte) in the three triploids suggested close homology among the three sets of chromosomes present.

Chromosome association in quadrivalents appeared to be common in the three triploids. It was not certain whether these were loose secondary associations, reported previously in blueberry (Jelenkovic and Hough 1970), or whether they were true multivalents resulting from translocation. The possibility has been raised by Ahokas (1971) and by Goldy (1983) that the basic chromosome number in Vaccinium might be 6 rather than 12 as has generally been assumed.

If $x=6$ in Vaccinium, quadrivalents and higher multivalents could be expected in a 36-chromosome plant where normal pairing relationships had been disrupted by wide hybridity.

The estimated unreduced gamete frequency for the three triploid clones was near 1%, and the very low fertility of the triploids in crosses with hexaploids was surprising. Two of the three triploids (Fla. 82-208 and Fla. 80-1) shed pollen rather copiously, and stigmas of the seed parents were heavily coated with pollen. It is likely that most stigmas received at least one ($3x=36$) gamete. Therefore, it appears that $3x$ gametes from the triploids were not very efficient at fertilizing $3x$ eggs from the hexaploids or from the triploids.

Because the number of flowers pollinated was great, a fairly large number of full-size seeds was obtained. In Vaccinium, $6x-3x$ and $3x-3x$ crosses, and $3x$ self-pollinations have not been previously reported. Chromosome numbers of progeny from these crosses suggest that a selective advantage exists for male gametophytes having approximately the same ploidy as the eggs. Most of the aneuploids from $3x-6x$ crosses had fewer than $2n=6x=72$ chromosomes. Evidently, female gametophytes from triploids may function even when they are deficient for more than one chromosome, whereas most aneuploid male gametophytes did not function.

It is hoped that by using these triploids to bridge diploid and tetraploid species, progeny can be selected which will combine the early fruit ripening of V. elliotii and V. corymbosum with the large berry size of V. corymbosum and V. ashei and the high vigor

and heat tolerance of V. *elliottii* and V. *ashei*. Studies on inheritance of these important characteristics are now underway.

CHAPTER IV

PRODUCTION AND EVALUTION OF SYNTHETIC HEXAPLOIDS IN VACCINIUM

Introduction

Vaccinium ashei Reade, rabbiteye blueberry ($2n=6x=72$), is one of three cultivated species in Vaccinium section Cyanococcus. The other two are tetraploids ($2n=4x=48$) V. corymbosum L. and lowbush V. angustifolium Ait. Rabbiteye blueberry is grown mostly in the southeastern United States but rabbiteye acreage is small compared to acreage of the tetraploid species. A number of factors are responsible for the limited expansion of the rabbiteye blueberry industry, such as later fruit ripening, a specific chilling requirement that limits its cultivation to a narrow region, and low winter hardiness.

The germplasm comprising the released V. ashei cultivars is based on a narrow genetic base consisting mainly of four wild selections (Lyrene 1983) which has made the species prone to inbreeding depression. Attempts have been made to broaden the genetic pool beginning with the intercrossing of V. ashei and V. corymbosum to transfer genes for early ripening and larger fruit from highbush to rabbiteye. The majority of progeny from these crosses are pentaploid (Chandler et al. 1985; Jelenkovic and Draper 1973; Moore et al. 1964). The pentaploids produced are partially

fertile, and progenies from backcrossing of the pentaploids to both V. ashei and V. corymbosum species tend to be less vigorous than the pentaploids.

Other sources of germplasm available for broadening the genetic base of the rabbiteye are the wild diploid species. Direct crosses between V. ashei and these diploids result mostly in pentaploids (Goldy and Lyrene 1984) and a small number of tetraploid seedlings (Darrow et al. 1949; Draper 1977). Crosses between tetraploid and diploid species give rise to tetraploid seedlings as a result of 2n gamete formation (Sharpe and Sherman 1971). Lyrene and Sherman (1983) were able to produce three triploid clones among tetraploid, pentaploid, and aneuploid progeny when they crossed tetraploid V. corymbosum to diploid V. elliotii Chapm. These triploids were very slightly fertile when crossed to V. ashei cultivars, and less than 0.01 hexaploid seedlings per pollination were produced as a result of unreduced gamete formation in the triploids (Chapter 3).

Chromosome doubling has been utilized to overcome crossing barriers between species (Dewey 1980). However, chromosome doubling of woody perennial species using colchicine is difficult (Darrow 1949; Derman and Bain 1941). Treatment in vitro has been successful for producing doubled blueberry plants. Perry and Lyrene (1984) obtained autotetraploid shoots by treating 3-node stem segments with 0.01% colchicine for two weeks in vitro.

Chromosome associations at meiosis in diploid and hexaploid Vaccinium species are predominantly bivalents (Longley 1927; Rousi 1966). Multivalents and secondary associations at diakinesis and metaphase I stages have been reported in the tetraploid species

(Jelenkovic 1970). Chromosomes in Vaccinium are extremely small (1.1-1.3 micron) and generally metacentric or submetacentric (Coville 1927; Hall and Galletta 1971). These features make the characterization of chromosome pairing in F1 progeny derived from homo- or heteroploid crosses difficult. Rousi (1966) noted a complete pairing in the F1 hybrids between two tetraploid species within Vaccinium and suggested that such pairing results from either a high degree of homology between genomes within the genus or autosyndetic pairing. Vorsa (1987) suggested that a minimum of 2/3 of V. ashei chromosomes can pair and recombine with V. corymbosum chromosomes in V. corymbosum/ashei first backcross derivatives, suggesting that interspecific genome homology may be significant.

Interspecific hybridization may be useful not only to broaden the genetic base, but also to study genome homology. The objectives of this report are to evaluate the feasibility of gene transfer from V. elliotii and V. corymbosum to V. ashei by doubling the chromosome number of a derived triploid (Lyrene and Sherman 1983; Chapter 3), and to study genomic relationships between the three species by using an F1 hybrid derived from hexaploid V. ashei crossed to the triploid.

Materials and Methods

Shoot-tip explants of triploid FL 81-19 (V. corymbosum X V. elliotii) (Lyrene and Sherman 1983; Dweikat and Lyrene 1988) were collected from actively growing shoots, immersed in 95% ETOH for one minute, transferred to a 1.3% sodium hypochlorite solution for twenty minutes, and rinsed three times in distilled sterilized water. Shoot tips of 2 cm length were transferred to 35-ml vials

containing 10 ml of blueberry micropropagation medium (Lyrene 1980) supplemented with 24.6 μ M 2ip (6- γ - γ -dimethylallyl amino purine). The vials were incubated at $22 \pm 2^\circ\text{C}$ under a 16-hr. photoperiod ($38\text{--}43 \mu\text{mol.s}^{-1} \cdot \text{m}^{-2}$ at the level of culture vials) using cool-white fluorescent bulbs. After eight weeks, 3-node stem cuttings from the newly proliferated shoots were placed horizontally in vials containing the same medium for a duration of three days. Five cuttings were placed in each of 70 vials.

The vials were divided into seven groups, each containing fifty explants. Group one was maintained as a control, whereas the others were transferred to a medium supplemented with 0.02% colchicine for intervals of six hrs. for total times of exposure to colchicine of six hrs. in one day, twelve hrs. in two days, eighteen hrs. in three days, twenty-four hr. in four days, thirty hrs. in five days, or thirty-six hr. in six days. Between colchicine treatments the explants were placed on colchicine-free medium.

After eight weeks, the cuttings were examined visually for shoots of unusually thick diameter, characteristic of chromosome doubling (Perry and Lyrene 1984). The thick shoots were cut into 3-node explants and used to establish new colonies. If thick shoots were produced from the daughter colonies, the shoots were rooted in peatmoss under intermittent mist. Shoot tips were examined microscopically to determine ploidy.

After these plants reached maturity, flower buds were collected and fixed in 1:1 ethanol:glacial acetic acid for twenty-four hr. at room temperature. The buds were then placed in fresh fixative and stored at -20°C . To study meiotic chromosome behavior, fixed buds

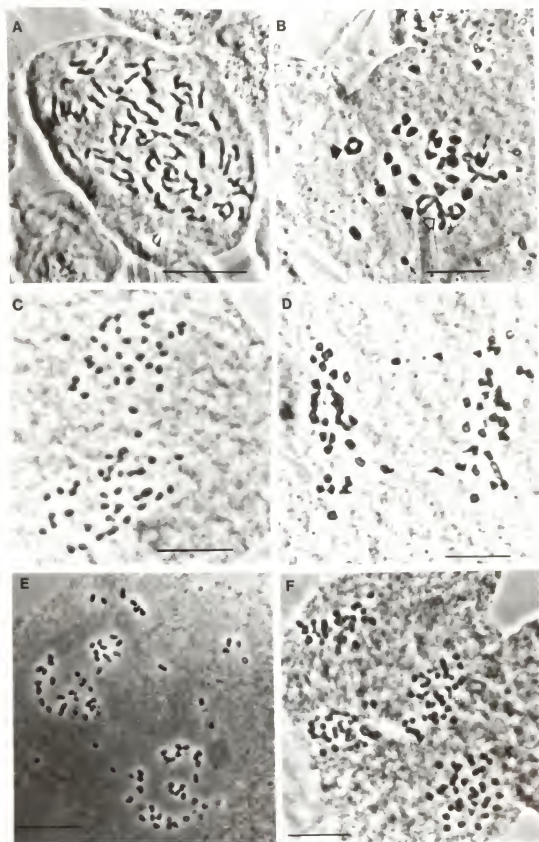
were rinsed in tap water and placed in about 10% pectinase in H₂O for twenty-four hr. to soften the tissue. Individual buds were separated and squashed in 1% acetocarmine and observed at 1000X using a phase contrast microscope. Flower buds were also collected from V. ashei clone 1, and from a hexaploid F1 hybrid between 'Powderblue' X FL 81-19 triploid (Chapter 3), designated Hex-F1, and treated as described previously for study of meiotic chromosome behavior.

Fertility of the synthetic hexaploid designated Hex-DT and the F1 hexaploid (Hex-F1) was evaluated by pollen stainability, pollen germination, and crossability to unrelated V. ashei clone (V. ashei clone 1). Crossability to V. ashei was defined as percent fruit set, seed per berry, and number of seedlings per pollinated flower. Pollen stainability was estimated by staining about 500 pollen grains from each clone using 1% acetocarmine for one hr. Percent pollen germination was measured using approximately 700 pollen grains from each clone on an agar medium supplemented with sugar and other nutrients (Goldy and Lyrene 1983).

Results

Six thickened shoots, two derived from a colchicine treatment of thirty hrs. over five days and four from a colchicine treatment of thirty-six hr. over six days, were produced. All had 72 chromosomes, twice the normal chromosome number of the triploid plant from which the cuttings were derived (Fig. 4-1 a). These doubled triploid plants, or synthetic hexaploids (Hex-DT), produced black fruit and dark green leaves characteristic of V. elliotii, rather than blue fruit and waxy blue-green leaves common to

Fig. 4-1. Mitotic and meiotic chromosomes in the synthetic hexaploid Hex-DT. A. somatic cell with 72 chromosomes. B. metaphase I with 23 II + 1 I (small arrow head) + 1 III (small arrow) + 1 IV (no arrow) + 3 VI (big arrow head). C. anaphase I with unequal chromosome disjunction, 34 (upper side): 38. D. anaphase I with 4 lagging chromosomes. E. anaphase I cell with unorganized chromosome disjunction. F. anaphase II with 34:34:38:38 chromosome distributions. (scale bar represent 10um).



hexaploid V. ashei.

Pollen stainability in the synthetic hexaploid plants averaged about 40% (Fig. 4-2 b) versus 88% and 91% in the V. ashei X triploid (Hex-F1) and V. ashei clone 1, respectively (Table 4-1 and Fig. 4-2 a). Pollen germination was 13%, 50.9%, and 52.0% in Hex-DT, Hex-F1, and in V. ashei clone 1, respectively (Table 4-1). The number of seedlings produced by Hex-F1 when pollinated by V. ashei clone 1 was not significantly different from V. ashei X V. ashei crosses, but was at least twice the number of seedlings produced by the synthetic hexaploid Hex-DT pollinated by V. ashei clone 1 (Table 4-2).

Chromosome behavior at meiosis in Hex-DT, Hex-F1, and in clone 1 differed. Chromosome associations at MI in the hexaploid V. ashei clone 1 consisted mainly of bivalents with a mean of only 0.42 quadrivalents per cell (29 PMC's) (Table 4-3 and Fig. 4-2 a). No lagging chromosomes or other abnormalities were observed. Metaphase I in Hex-DT was irregular, with more than 40% of the chromosomes in 27 PMC's involved in non-bivalent formations, including hexavalents, quadrivalents, trivalents, and univalents (Table 4-3 and Fig. 4-3 b). Anaphase I, was studied in 31 PMC's and 75% showed from 1 to 5 lagging chromosomes (Figs. 4-3 d and 4-3 e). Other abnormalities were also observed in anaphase I. Abnormalities such as unequal disjunction and numerically unbalanced distribution of chromosomes were observed in 80% of the PMC's examined (Fig. 4-3 c). These abnormalities resulted in unequal chromosome distribution in nearly 80% of the PMC's observed at anaphase II.

The hexaploid F1 hybrid between V. ashei and the triploid displayed fewer abnormalities than the doubled triploid. The hybrid

Fig. 4-2. Comparison of pollen from V. ashei clone 1 and Hex-DT.
A. normal staining pollen from clone 1. B. much aborted and
irregular pollen from Hex-DT.

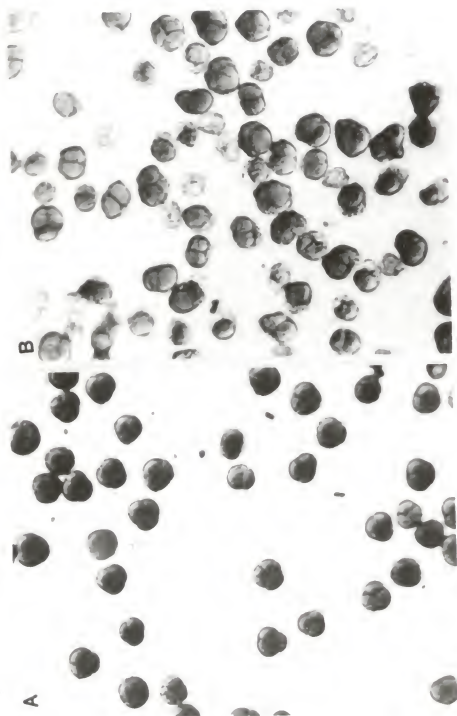


Table 4-1. Pollen stainability and germination of Hex-Dt, Hex-Fl, and V. ashei clone 1.

Clone	Stainability	Germination
	(%)	(%)
Hex-DT ^z	40.2 a ^w	13.0 a
Hex-Fl ^y	87.9 b	50.9 b
<u>V. ashei</u> clone 1 ^x	91.0 b	52.0 b

^z Synthetic hexaploid derived by colchicine doubling of the triploid hybrid obtained by crossing tetraploid V. corymbosum X diploid V. elliotii.

^y A hexaploid hybrid from the cross of hexaploid V. ashei cultivar 'Powderblue' X triploid hybrid FL 81-19.

^x Vaccinium ashei line.

^w Mean separation within columns by Duncan's multiple range test, 5% level.

Table 4-2. Crossability of Hex-DT and Hex-F1 to V. ashei (clone 1).

Parental clone	No. flowers pollinated	Fruit set (%)	No. seed/ berry	No. seedling/ flower
Hex-DT X clone 1	220	59.3	14.6	5.9 a ^y
clone 1 X Hex-DT	237	40.5	9.7	5.0 a
Hex-F1 X clone 1	400	73.0	18.3	10.9 b
clone 1 X Hex-F1	370	69.8	17.6	10.7 b
clone 1 X F 87-50 ^z	100	74.2	18.7	11.2 b

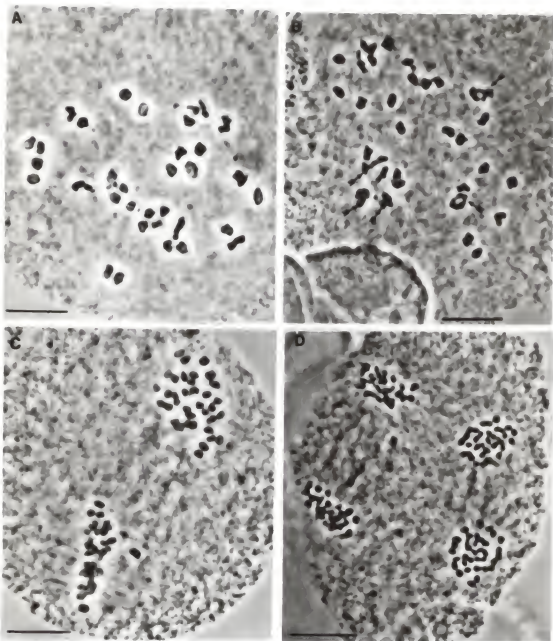
^z Vaccinium ashei line.^y Mean separation by Duncan's multiple range test, 5% level.

Table 4-3. Chromosome associations at metaphase I in Hex-DT, Hex-F1, and clone 1.

Chromosome associations at MI							
Clone	No. of cells examined						Bivalents (%)
		Uni valents	Bi- valents	Tri- valents	Quadri- valents	Hexa- valents	
Hex-DT	36	0-2 ^z (1.31) ^y	14-23 (20.30)	0-4 (2.31)	2-4 (2.93)	1-4 (2.11)	54.7
Hex-F1	71	0 (0)	23-31 (29.14)	0-2 (0.87)	1-3 (1.91)	0-2 (0.58)	80.9
clone 1	29	0 (0)	32-36 (35.16)	0 (0)	0-2 (0.42)	0 (0)	97.7

^z Range among cells.^y Mean for all cells.

Fig. 4-3. Meiotic chromosome associations in *V. ashei* clone 1 and in the F1 hybrid Hex-F1. A. metaphase I in clone 1 with 36 II. B. metaphase I in Hex-F1 with 23 II + 5 IV (small arrow) + 1 VI (big arrow). C. anaphase I in Hex-F1 with no lagging chromosomes. D. anaphase II with two lagging chromosomes. (scale bar represent 10 um).



(Hex-F1) had an average of 27.4 bivalents per cell in 71 PMC's observed. Multivalents accounted for less than 20% of the total number of chromosomes observed in 71 PMC's. Hexavalents, quadrivalents, and trivalents were observed at means of 0.58, 1.91, and 0.87 per cell, respectively (Table 4-3). Anaphase I cells displayed normal chromosome disjunction with only 2 of 29 PMC's showing lagging chromosomes. Anaphase II cells observed were mostly regular.

Discussion

In the present study, treatment of shoot segments with 0.02% colchicine for six days at six hrs. per day was most effective for producing doubled plants. Perry and Lyrene (1984) found 0.01% colchicine in a solid medium for two weeks of continuous exposure to be most effective. In this experiment a six hrs. duration was chosen in order to give the dividing cells a chance to recover from the toxic effect of colchicine.

The derived synthetic hexaploid plants showed a remarkable increase in pollen stainability over the original triploid, which had less than 2% stainability (Chapter 3). Lack of uniformity of pollen in the doubled triploid (Fig. 4-2 b) was mainly due to microspore aneuploidy, which resulted from lagging chromosomes and unequal disjunction during anaphase I and II. On the other hand, the F1 hexaploid hybrid (V. ashei X triploid) showed more regular meiosis and produced pollen with high stainability and germination. The number of seedlings produced by Hex-DT pollinated with V. ashei was over 300 times higher than the number produced by the original highly sterile triploid. One reason for polyploidy induction is

enhancement of fertility in order to facilitate interspecific gene transfers (Dewey 1980). The lower pollen viability of the doubled triploid (Hex-DT) clone when compared with the F1 hexaploid (Hex-F1) was mainly due to the high a multivalent frequency (45.3%) which resulted in irregularities such as lagging chromosomes and unequal disjunction at anaphase I and II. This would give rise to gametes with an aneuploid chromosome number. The F1 hexaploid displayed a lower frequency of multivalents with very few irregularities during meiosis.

The F1 hybrid (Hex-F1) obtained from V. ashei X triploid as a result of unreduced gamete formation by the triploid, is a composite of 3 species with 3/6 of the genome derived from V. ashei, 2/6 from V. corymbosum, and 1/6 from V. elliotii. High pollen viability and meiotic regularity in Hex-F1 is an indication of strong genome homology among these three species. Vorsa (1987) suggested a high degree of homology between 2/3 of the V. ashei genome with that of V. corymbosum. High fertility and vigor in F1 tetraploids derived from V. corymbosum X V. elliotii (Lyrene unpublished) also suggest evidence of homology between these species.

Due to the similarity in appearance of chromosomes in Vaccinium (Hall and Galletta 1971), pairing relationships are not easy to determine. However, the high fertility in the F1 Hex-F1 suggests that autosyndetic pairing occurs (Lyrene and Ballington 1987; Rousi 1967, Vorsa 1987).

The results of this study suggest that the transfer of genes between these three species is feasible. The main barrier to gene

flow between species of different ploidy levels in Vaccinium may be due to the differences in ploidy rather than to low genome homology.

CHAPTER V

MORPHOLOGY, CYTOLOGY, AND BREEDING BEHAVIOR OF INDUCED AUTOTETRAPLOIDS OF VACCINIUM ELLIOTTII.

Introduction

In section Cyanococcus of Vaccinium there are nine diploid ($2n=2x=24$), twelve tetraploid ($2n=4x=48$), and three hexaploid ($2n=6x=72$) species (Camp 1945; Darrow et al. 1944). Genes for important horticultural traits exist at all 3 ploidy levels; each ploidy level has useful genes not found at the others (Moore 1965). Cultivated forms exist at the tetraploid and hexaploid levels, but diploid Cyanococcus species are uncultivated.

Vaccinium elliotii Chapm. is a diploid found from Florida to Virginia and west to Texas. This species is generally low chilling, early ripening, tolerant of dry upland soils, and disease resistant. Its fruit are very small but otherwise of medium to high quality (Lyrene and Sherman 1980). Hybrids between V. elliotii and the cultivated highbush blueberry, V. corymbosum L., are difficult to obtain. These hybrids consist of tetraploids, triploids, pentaploids, and aneuploids (Lyrene and Sherman 1983).

One reason this cross is hard to make is the existence of a strong triploid block (Marks 1966), a phenomenon not unique to blueberry (Woodell and Valentine 1961). One way to overcome this block is to double the chromosome number of the diploid species. Draper et al. (1972) used colchicine to double the chromosome number

of V. atrococcum Heller ($2n=24$) in order to transfer its resistance to Phytophthora cinnamomi Rands to tetraploid highbush cultivars.

Chromosome doubling of diploids has been used in many plant species (Dewey 1979; Sanford 1983). The resulting autotetraploids showed changes in morphological features, chromosome behavior at meiosis and fertility. In V. atrococcum, larger leaves, flowers and pollen resulted from autotetraploidy as well as a slight breakdown in self-incompatibility (Draper et al. 1972).

Cytological studies in Vaccinium have revealed generally regular pairing in the diploid species (Longely 1927), but a few pairing irregularities have been reported in tetraploids (Jelenkovic and Hough 1970). This study was carried out to determine the effect of chromosome doubling on plant morphology, chromosome behavior, and fertility of two autotetraploid V. elliotii plants produced by in vitro colchicine treatment (Perry and Lyrene 1984).

Materials and Methods

The plant materials used in this study consisted of two clones, Fla. 156 and Fla. 519, derived by in-vitro colchicine treatment of 2-node cuttings from seedlings of V. elliotii ($2n=24$) (Perry and Lyrene 1984). The tetraploid highbush (V. corymbosum) cultivar O'Neal, one native diploid ($2n=24$) clone of V. elliotii selected for its high fertility, and one clone of V. darrowi ($2n=24$), were also used in the crosses. The two V. elliotii clones derived from the colchicine treatment were determined to be tetraploid by chromosome counts of somatic cells.

Before the flowering season, the plants were chilled at 7°C for six weeks and then transferred to a greenhouse. The two

autotetraploid clones were intercrossed, self-pollinated, and reciprocally crossed to both the cultivated highbush and the diploid V. elliotii. For comparison, the two diploid species (V. elliotii and V. darrowi) were intercrossed (Table 5-2). The plants used in this study were all about four years old.

Pollen viability was evaluated by in vitro pollen germination on an agar medium supplemented with sugar and other nutrients (Goldy and Lyrene 1983). Pollen stainability was measured using 1% acetocarmine for one hour. Scanning electron microscopy was also used to determine pollen size and pollen irregularities.

Flower buds from Fla. 156, Fla. 519, and the diploid V. elliotii clone were fixed in 1:1, ethanol:glacial acetic acid for 24 hours at room temperature. The buds were then placed in fresh fixative and stored at -20°C. To study meiotic chromosome behavior, fixed buds were rinsed in tap water and placed in 10% pectinase solution for 24 hours to soften the tissue. Individual flower buds were separated and squashed in 1% acetocarmine and observed at 400X and 1000X with a phase contrast microscope.

Leaf area was determined from ten fully expanded leaves from each of the autotetraploid clones and from the diploid V. elliotii using a Li-3000 Li-cor portable leaf area meter. Twenty random berries and 200 seeds were weighed from each clone (Table 5-1). The seeds were extracted by hand from ripe berries, and large, well-filled seeds were counted. The seeds were air-dried and stored in paper bags at 5°C for six months, and then sown on peatmoss under intermittent mist in the greenhouse. Germination percentage was determined before seedlings were transferred to 50x20 cm flats.

Results

Morphology

Autotetraploid V. elliotii produced more compact growth, darker leaf color, thicker stems and larger flowers than diploid. The leaves of the autotetraploids were over 70% larger and had coarser venation (Table 5-1, Fig. 5-1 a), with clone Fla. 156 having larger and thicker leaves than clone Fla. 519. Stomatal guard cells of the autotetraploids averaged 18% longer than those of the diploid (Table 5-1, Figs. 5-1 c and 5-1 d). Pollen diameter was 37% larger in the autotetraploids (Table 5-1, Figs. 5-1 e and 5-1 f) and fruit and seed weights were also increased (Table 5-1, Fig. 5-1 b).

Fertility

Pollen viability, estimated as percent stainable pollen and pollen germination, averaged 10 and 40% lower, respectively, in the two autotetraploids than in the diploid (Table 5-1). Clone Fla. 156 had slightly higher pollen viability than clone Fla. 519. In crosses it was higher in male fertility but lower in female fertility (Table 5-2). Overall fertility was measured as percent fruit set, average number of seeds per fruit, and number of seedlings per pollination. Clone Fla. 519 produced more seedlings per pollinated flower when used as a female parent than when used as a male parent, both in crosses with highbush cultivar O'Neal and in crosses with Fla. 156 (Table 5-2). The two autotetraploid V. elliotii clones produced about half as many seeds per pollinated flower when pollinated with pollen from tetraploid V. corymbosum as did diploid V. elliotii pollinated by diploid V. darrowi.

Intercrossing the two autotetraploid clones produced an average

Table 5-1. Average phenotypes for autotetraploid and diploid clones of V. elliotii.

Clone	Ploidy level	Leaf area (cm ²)	Guard cell length (μm)	Leaf thickness (μm)	Pollen diameter (μm)	Seed weight (mg)	Fruit weight (g)	Pollen stainability (%)	Pollen germination (%)
Fla. 156	4x	3.93 ^z	210 a	225 a	44.4 a	0.87 a	0.66 b	83.0 b	37.7 b
Fla. 519	4x	3.06 b	201 a	206 b	43.0 a	0.78 b	0.75 a	77.1 c	31.9 c
<u>V.elliotii</u> 2x		1.95 c	168 b	183 c	31.7 b	0.62 c	0.30 c	87.9 a	58.6 a

^z Mean separation within columns by Duncan's multiple range test, 5% level.

Fig. 5-1. Morphological characters of diploid and autotetraploid V. elliotii. (a) branches of autotetraploid (left) and diploid (right). (b) fruits of autotetraploid (left) and diploid (right). (c) stomata gurd cells of diploid. (d) stomata gurd cells of autotetraploid. (e) autotetraploid pollen. (f) diploid pollen. Scale = 200 um.

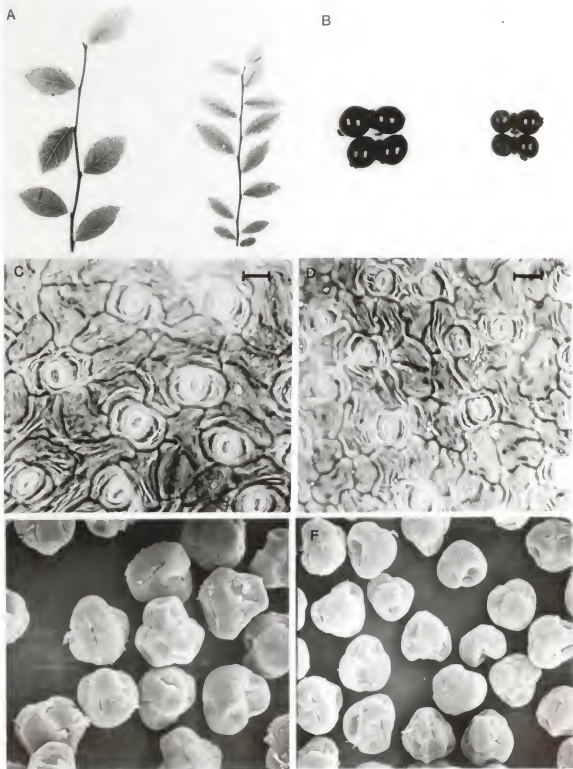


Table 5-2. Crossability and fertility data for two autotetraploid *V. elliotii* clones.

Cross		No. of flower pollinated	Fruit set (%)	Avg. no. seeds per fruit	No. seedlings per pollination	No. seedlings per pollination
Female	Male					
Fla. 519 (4x)	O'Neal ^z (4x)	118	62.0	15.5	850	7.21
Fla. 156 (4x)	O'Neal (4x)	95	48.4	13.6	472	4.97
O'Neal (4x)	Fla. 519 (4x)	185	53.0	7.7	528	2.86
O'Neal (4x)	Fla. 156 (4x)	103	78.0	14.6	801	7.77
Fla. 519 (4x)	Fla. 156 (4x)	100	72.0	7.3	213	2.13
Fla. 156 (4x)	Fla. 519 (4x)	82	34.0	7.7	128	1.56
O'Neal (4x)	Sharpblue ^z (4x)	50	86.0	18.3	613	12.27
Fla. 156 (4x)	<i>V. elliotii</i> (2x)	1389	0.0	0.0	0.0	0.0
Fla. 519 (4x)	<i>V. elliotii</i> (2x)	1120	0.0	0.0	0.0	0.0
<i>V. elliotii</i> (2x)	Fla. 156 (4x)	780	6.1	1.3	29 ^x	0.04
<i>V. elliotii</i> (2x)	Fla. 519 (4x)	585	0.0	0.0	0.0	0.0
Fla. 156 (4x)	Self-pollinated ^w	940	0.0	0.0	0.0	0.0
Fla. 519 (4x)	Self-pollinated	892	0.0	0.0	0.0	0.0
<i>V. elliotii</i> (2x)	Self-pollinated	650	4.3	1.7	35	0.05
<i>V. elliotii</i> (2x)	<i>V. darrowi</i> ^y (2x)	50	89.0	17.8	594	11.88

^z Two tetraploid cultivars.^y A diploid species highly cross-compatible with *V. elliotii*.^x Twenty seven plants were tetraploid.^w Manual selfing.

of 1.85 seedlings per pollination, whereas a cross between two highbush cultivars (O'Neal X Sharpblue) gave 12.27 seedlings (Table 5-2). When clone Fla. 156 was backcrossed, as pollen donor, to the diploid V. elliotii, twenty-nine seedling were produced from 780 pollinated flowers, and nearly 90% of these were tetraploid. The reciprocal cross produced no backcross seedlings, and clone Fla. 519 produced no seedlings in crosses with diploid V. elliotii in either direction. Self-incompatibility in the autotetraploids was not weakened by chromosome doubling, but appeared to be even stronger than in the diploid (Table 5-2).

Cytological behavior

Meiotic chromosomal behavior in diploid V. elliotii was normal in 42 cells examined (Figs. 5-2 a and 5-2 c). In both autotetraploids, univalents, bivalents, trivalents, and quadrivalents were found at diakinesis and metaphase I (Table 5-3, Figs. 5-2 b and 5-2 d). In Fla. 519 a higher frequency of univalents, trivalents, and quadrivalents was observed at diakinesis and metaphase I than in clone Fla. 156 (Table 5-3). The mean frequency of chromosome associations other than bivalents was higher at diakinesis than at metaphase I. Multivalents higher than quadrivalents were absent or rare, but abnormalities such as clumping of chromosomes at metaphase I were often observed.

At anaphase I, 56.3 and 68.9% of the cells from clones Fla. 519 and Fla. 156 respectively, had equal chromosome disjunction (Table 5-4). At anaphase I 32.1% of the cells of Fla. 156 and 44.7% of the cells of Fla. 519 1-2 laggards (Table 5-4, Fig. 5-2 e). By anaphase II, most of the cells examined showed normal chromosome distribution

Fig. 5-2. Chromosome associations at diakinesis and metaphase I in diploid V. elliotii and in Fla. 519. a. Diakinesis in the diploid with 12 II. b. Diakinesis in Fla. 519 with IV (big arrow head) + 3 III (long arrow) + 1 I (small arrow head) + 9 II. c. Metaphase I in the diploid with 12 II. d. Metaphase I in Fla. 519 with 3 IV (big arrow head) + 3 III (long arrow) + 1 I (small arrow head). e. Anaphase I with 24 +22 and 2 lagging chromosomes. f. Normal anaphase II in Fla. 519. Scale = 5 μ m.

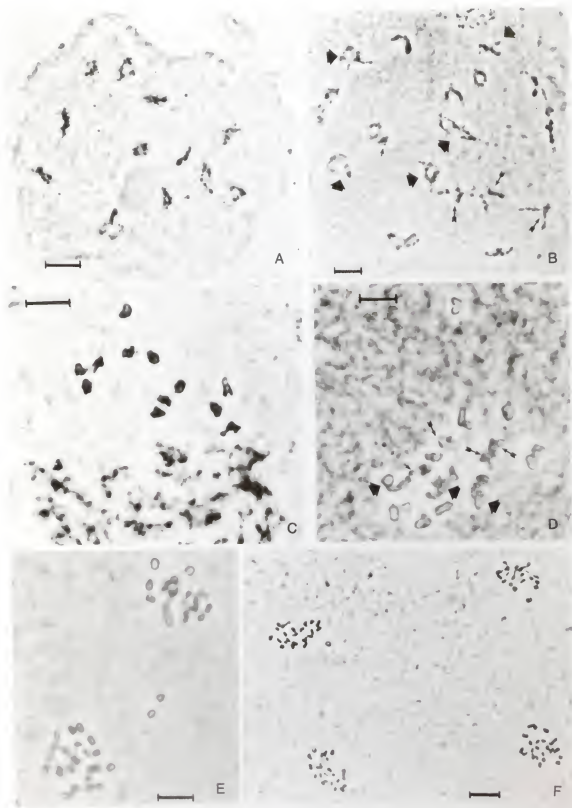


Table 5-3. Range and mean of chromosome associations at diakinesis (DK) and metaphase I (M) in autotetraploids of V. elliotii.

Clone	Total no. of cells	Stage of meiosis	Stage of			
			I	II	III	IV
Fla. 156	22	DK	0 - 2	10 - 14	0 - 3	2 - 5
			(0.66)	(12.76)	(2.17)	(3.89)
	13	M	0 - 2	12 - 16	0 - 2	2 - 4
			(0.45)	(14.39)	(1.69)	(3.43)
Fla. 519	27	DK	0 - 3	9 - 14	0 - 3	2 - 5
			(0.96)	(11.62)	(2.36)	(4.18)
	9	M	0 - 2	10 - 15	0 - 3	2 - 5
			(0.71)	(13.11)	(1.75)	(3.82)

Table 5-4. Chromosome distributions at anaphase I in 136 cells of the two autotetraploids.

Anaphase I distribution	No. of cells		(Z)	
	Fla. 519	Fla. 156	Fla. 519	Fla. 156
24 - 24	34	51	56.3	68.9
24 - 23 - 1 ^z	12	9	18.4	12.2
25 - 22 - 1	7	5	11.7	6.7
26 - 21 - 1	4	3	5.8	4.1
24 - 22 - 2	5	6	7.8	8.1
Total	62	74	100.0	100.0

^z Lagging chromosome.

(Fig. 5-2 f), with the exception of three of twenty-one cell from clone Fla. 519, which showed lagging chromosomes.

Discussion

The morphological changes observed in induced polyploids in V. elliotii, such as increased leaf area and leaf thickness, coarser venation, compact growth, and larger pollen and fruit were similar to the effects reported previously in Vaccinium (Draper et al. 1972; Moore et al. 1964) and in many other species (Dewey 1979; Sanford 1983). The most important morphological feature resulting from chromosome doubling was the increase in fruit size, since small fruit is a major factor preventing cultivation of diploid Vaccinium species (Sanford 1983).

The reduced pollen viability of the two autotetraploid V. elliotii clones compared to diploid V. elliotii may be due in part to abnormal cytological behavior of the pollen mother cells, as observed in this study, or to genetic factors. Munoz and Lyrene (1987) reported differences in pollen viability among selections of native V. elliotii. Levels of male and female fertility differed between the two autotetraploids. Although meiosis was not studied in megaspore mother cells, it is likely that the differences observed in male and female fertility were due to genetic factors (Munoz and Lyrene 1987) or physiological and genetical factors combined (Stebbins 1947) rather than cytological irregularities.

In most gametophytic incompatibility systems, chromosome doubling has been found to weaken or eliminate the incompatibility (Lewis 1954; Mark 1966; Nettancourt 1969; Pandey 1958; Sebastiampillai and Jones 1977). This was not the case in the

autotetraploids of V. elliotii, suggesting that the incompatibility in Vaccinium is not gametophytic (El-Agamy 1979).

Since meiosis was normal in the diploid studied, the cytological abnormalities at meiosis in the autotetraploids were regarded as a consequence of chromosome doubling. The presence of univalents, trivalents, and quadrivalents at diakinesis and metaphase I is a phenomenon seen in doubled diploids of many species (Dermen 1938; Jackson and Casey 1979). The decrease in trivalent and quadrivalent frequencies from diakinesis to metaphase I was also observed in autotetraploids of Fragaria (Sebastiamplai and Jones 1977)

Induced tetraploids in Vaccinium may prove to be of significant value in breeding, by facilitating gene exchange between the diploid and tetraploid species. Important traits exist in both diploid and tetraploid species and direct tetraploid X diploid crosses give rise to a low frequency of tetraploids along with triploids, pentaploids, and aneuploids (Lyrene and Sherman 1983). Doubling the chromosome number of the diploids eliminates or greatly reduces the crossing barrier between diploid and tetraploid Cyanococcus species. The ease of crossing the species at the tetraploid level suggests a close relationship between V. elliotii and V. corymbosum, and indicates that the crossing barrier between these species is due largely to the difference in ploidy level and not to genic incompatibility.

CHAPTER VI

USE OF TWIN SEEDLINGS FOR THE PRODUCTION OF HAPLOID PLANTS IN VACCINIUM SPP.

Introduction

Haploids in higher plants can originate spontaneously or by induction. Haploid sporophytes are found at various frequencies in seedling populations. Spontaneous haploids have been reported in flax (Kappert 1933), maize (Chase 1949), potato (Hougas et al. 1964), and cotton (Turcotte and Feaster 1969). Numerous examples of multiple embryo formation are recorded in the literature for many species. The multiple embryos within a seed can be of variable origin and different ploidy (Masheswari and Sachar 1963).

The method of screening for twin seedlings and selection of haploids has been successful in at least forty-two plant species (Lacadena 1974). The frequency of observed polyembryony is extremely low and species-dependent; for instance, the frequency was reported at 4% in soybean (Kenworthy 1973), 0.37% in Capsicum (Morgan and Rappleye 1950), 0.10% in maize (Sarker and Coe 1966), and 0.054 in grape (Bouquet 1980). Twin seedlings usually show one of the following combinations: diploid-diploid, diploid-haploid, haploid-haploid, and diploid-triploid (Lacadena 1974).

The probable origin of haploids in these twins has been discussed thoroughly by Lakshmanan and Ambegaokar (1984). The haploid member of the twins is often morphologically distinct,

slower in growth, and much smaller than the diploid member (Hesse 1971; Morgan and Rappleye 1950; Randall and Rick 1945; Thompson 1977; Toyama 1974; Wilson and Ross 1961).

Gene markers have been used to determine the probable origin of twins. For instance, Thompson (1977) used the flax rust (Melampsora lini) resistance marker gene to demonstrate that the haploid member of a set of twin of maternal origin. Morgan and Rappleye (1954) used two varieties of Capsicum frutescens, differing in fruit pungency, immature fruit color, and fruit shape in reciprocal crosses and determined that the haploids were of maternal origin.

Haploids are useful in plant breeding for producing immediate homozygosity following chromosome doubling. Haploids have also been used to facilitate gene transfer from diploid to tetraploid lines in potato (Pelouquin et al. 1966). The ability to reduce chromosome number has also been useful in the study of evolutionary relationships between plant species (Howard 1973).

Haploids in blueberry have not been reported, and an attempt to produce haploids using anther culture was not successful (Lyrene 1978). The objectives of this study were to screen germinating seed from different Vaccinium species for twin seedlings, to identify any haploids derived from the twins, and to attempt to determine the origin of the twins by using an anthocyanin deficient mutant.

Materials and Methods

Blueberry fruits were harvested following open-pollination of clones from Vaccinium ashei (6x), V. corymbosum (4x), V. elliotii (2x), and V. darrowi (2x). Seeds were extracted using a Waring blender and air dried at room temperature for two days. Seeds were

spread over sphagnum peatmoss in 10-cm pots and placed under intermittent mist in a half-shaded greenhouse. When the radicals emerged and reached 1cm in length, seedlings were counted and removed and screened for twins.

A second experiment was conducted to explore the possibility of germinating the seed under sterile conditions in vitro. Hexaploid seeds from the same lot were soaked in 4000 ppm gibberellic acid (GA_3) at 22°C for twenty-four hr. At the end of the treatment period, seeds were surface disinfected with 1.6% sodium hypochlorite for twenty minutes, rinsed three times in sterile distilled water and spread in 9-cm petri dishes containing 0.6% agar. The plates were placed on a window sill to receive full sunlight. Germinated seeds were counted and removed and screened for twins. Twin seedlings were transferred to sterile 35ml vials containing agar media. Shoot tip squashes were performed for all twin individuals found in both experiments and examined microscopically to determine ploidy.

A third experiment was conducted to determine the origin of twins found in the diploid V. elliotii. A V. elliotii mutant, deficient for anthocyanin in foliage, buds, and fruits, was crossed as seed parent to a normal diploid V. darrowi (selected to avoid self incompatibility). Anthocyanin deficiency in this mutant is conditioned by a single recessive gene (Lyrene 1988). The F1 seeds were harvested and germinated as described previously. Twins were exposed to 9°C temperature for fourteen hours a day for ten days to stimulate anthocyanin production.

Results

At germination, twin and triplet seedlings were identified by the emergence of two or three radicals from one seed (Fig. 1-6 a). The frequency of twinning among the seeds germinated under greenhouse conditions is shown in Table 6-1. In screening 86,000 seedlings from four species and three ploidies of Vaccinium , fifty-three pair of twins and one triplet were found. All twins were unattached to one another and no conjoined twins were observed. The frequency of twins was highest in the hexaploid and lowest in the diploids (Table 6-1).

About 40% of the twin pairs consisted of a strong and a weak individual (Fig. 6-1 b). Growing twin seedlings under greenhouse conditions produced poor results in terms of survival of the weaker member of the twins. Only the vigorous members survived to be examined cytologically, and each carried a normal $2n$ chromosome number (Table 6-2).

No relationship was observed between seed size and the production of twins. However, most of the twins observed were found in later germinating seed. In fact, most seed germinated after three weeks, yet the majority of twins were produced from seed that germinated after four weeks.

Screening of about 15,000 hexaploid seedlings germinated in vitro revealed thirteen pairs of twins (0.87% frequency). Among these thirteen twins three were in the combination of triploid-hexaploids, as determined by chromosome counts (Figs. 6-1 e and 6-1 f). The triploid (trihaploid) member was smaller in size as a result of slower growth rate (Fig. 6-1 b). The remaining twins

Fig. 6-1. Characterization of twins obtained from hexaploid V. ashei species. A. germinated seed with a two radicals emerging. B. twin seedlings consisted of trihaploid (right), and hexaploid (left). C. twin seedling consisting of two hexaploids. D. two hexaploid monoembryonic seedlings. E. somatic cell with 36 chromosome from trihaploid member of a twin. F. somatic cell with 72 chromosomes from a hexaploid member of a twin.

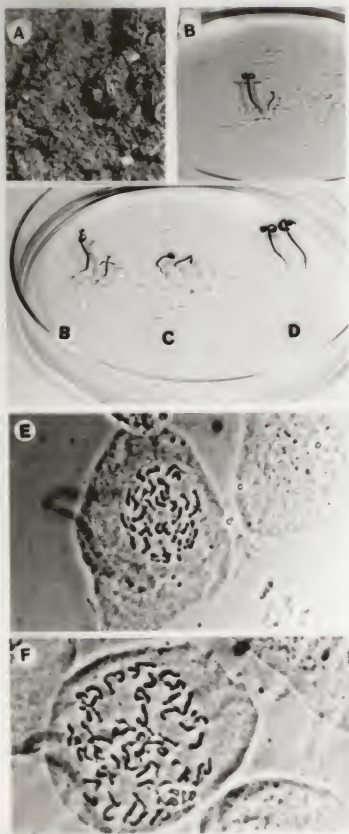


Table 6-1. Number and frequency of twins in different species of Vaccinium.

Species	No. of		No. of sets of twins	Frequency ^z (%)
	ploidy	seedlings		
<u>V. ashei</u>	6x	38,000	28	0.074
<u>V. corymbosum</u>	4x	30,000	20	0.066
<u>V. elliotii</u>	2x	14,000	5	0.036
<u>V. darrowi</u>	2x	3,000	1	0.033

^z Number of seedlings from polyembryony divided by total number of seedlings.

Table 6-2. Determination of ploidy in twin individuals in 4 species of Vaccinium germinated in the greenhouse.

Seed source		Number of pairs of twins with ploidy		
Species	Ploidy	2n:2n	2n:? ^z	undetermined ^y
<u>V. ashei</u>	6x	17	8	3 ^x
<u>V. corymbosum</u>	4x	13	5	2
<u>V. elliotii</u>	2x	3	2	
<u>V. darrowii</u>	2x	1		

^z Only one member of the twins survived.

^y Both members of the twins died.

^x Two sets of twins, 1 set of triplets.

were hexaploid-hexaploid and produced seedlings of approximately equal size (Fig. 6-1 c), but still smaller than monoebryonic seedlings (Fig. 6-1 d)

In screening 9,100 germinated F1 seeds, produced by pollinating 1000 flowers of the anthocyanin-deficient V. elliotii mutant, three twin pairs were found. Two pairs gave rise to individuals of equal size, and one produced a weak and strong seedling. Anthocyanin was produced by all the strong individuals, but not by the weak one. The chromosome number in the weak seedling was not determined.

Discussion

On the basis of this study, the average frequency of twins, or polyembryony, in blueberry (Vaccinium spp.) can be estimated at 0.067%. To our knowledge polyembryony in blueberry has not been reported previously.

Screening germinated seed under greenhouse conditions may not provide accurate estimation of twinning frequency when compared to germination in vitro. Twins in the greenhouse under misting are subject to more unfavorable conditions which may reduce survival, especially of the weaker individual, before they are observed. In vitro, smaller numbers of seed are germinated in each container under controlled and favorable conditions, giving a more accurate estimate of twinning frequency.

Although twin seedlings could be identified at early stages of germination, the seed which gave rise to twins was not distinctive in appearance. No chromosome counts were performed on the weak members of twins isolated in the greenhouse, due to low survival rate. It seems likely that most, if not all, were haploids based

on chromosome counts of the weak individuals produced in vitro. Weak members of twins, which exhibit slower growth rate and smaller size, have been determined to be haploids in many plant species (Hesse 1971; Morgan and Rappleye 1950; Randall and Rick 1961; Thompson 1973; Toyama 1974; Wilson and Ross 1961).

Species differed in the frequencies of twin seedlings (Table 6-1). Twin seedlings arise at least two times as frequently in the hexaploid and tetraploid species as in the diploid. The seed used in this study was collected from plants grown under relatively uniform environmental conditions, therefore it is likely that the difference in twinning frequencies is due mainly to genotype rather than to environmental variation.

The frequency of twins is often so low that it is difficult to determine their exact origin by developmental studies; however, one can speculate on their origin based on cytological and genetical studies (Lakshmanan and Ambegaokar 1984). Most of the $2n-2n$ surviving twins showed no difference in their morphological features, which suggests that they may originate by cleavage polyembryony at an early developmental stage (Lakshmanan and Ambegaokar 1984; Morgan and Rappleye 1950). This is supported by observation of unattached twin members, and by the results of crosses between the anthocyanin-deficient mutant and the wild type. In this experiment, the twins which gave rise to $2n-2n$ individuals showed the phenotype of the wild type parent. The weak individual from the third pair of twins was anthocyanin-deficient. The production of anthocyanin in the twins indicates that the individuals received one dominant allele for anthocyanin production

from the male parent. The lack of anthocyanin production in the weak member of the third set of twins indicates that this individual received only the recessive allele contributed by the female parent.

The observation of polyembryony and the production of haploids should be helpful in facilitating gene transfer between different ploidies in Vaccinium. In addition, we hope to use this strategy to characterize species relatedness and to better understand the events which took place in polyploidization of Vaccinium species.

CHAPTER VII

CONCLUSIONS

Although triploid clones derived from 4x-2x interspecific hybridization were highly sterile, hexaploid plants were produced as a result of unreduced gamete formation or colchicine treatment. In one case hexaploids were derived by 6x-3x hybridization and had a genome consisting of 3/6 V. ashei, 2/6 V. corymbosum, and 1/6 V. elliottii. In the second case, the hexaploid was derived by chromosome doubling and therefore, the genome consisted of 2/3 V. corymbosum and 1/3 V. elliottii. Both strategies should be valuable in enhancement of genetic variability available for improvement to the cultivated hexaploid V. ashei.

Doubling the chromosome number of both triploid and diploid clones resulted in multivalent formation at metaphase I and lagging chromosomes at anaphase I and II. On the other hand, it reduced crossing barriers which exist between diploid and tetraploid species, and between diploid, tetraploid, and hexaploid species. This study demonstrates that the transfer of genes among diploid, tetraploid, and hexaploid species of Cayanococcus is feasible. The main barrier to gene flow among these species may therefore have been principally due to the differences in ploidy rather than to low genome homology. It is hoped that use of these triploids, and their derived hexaploids, to bridge diploid, tetraploid, and hexaploid species will facilitate

the combining of early fruit ripening, from V. *elliottii* and V. *corymbosum*, large berry size from V. *corymbosum* and V. *ashei*, with high vigor and heat tolerance, from V. *elliottii* and V. *ashei*.

The screening of germinated seeds for twins was a successful method for producing polyhaploid seedlings in Vaccinium. Seed germination and screening in vitro resulted in a higher survival rate when compared to screening under greenhouse conditions. The derived polyhaploids may be used to produce homozygous lines which may ultimately be useful in development of homozygous, heterotic F₁ hybrids. In addition, characterization of these haploids may facilitate the study of evolutionary relationships among species of different ploidy.

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.



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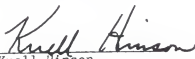
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